# ADS1 encodes a MATE-transporter that negatively regulates plant disease resistance

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# *ADS1* encodes a MATE-transporter that negatively regulates plant disease resistance

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## Summary

• Multidrug and toxic compound extrusion (MATE) proteins comprise the most recently identified family of multidrug transporters. In plants, the numbers of MATE proteins has undergone a remarkable expansion, underscoring the importance of these transporters within this kingdom.

• Here, we describe the identification and characterization of *Activated Disease Susceptibility 1* (*ADS1*) which encodes a putative MATE transport protein. An activation tagging screen uncovered the *ads1-Dominant* (*ads1-D*) mutant, which was subsequently characterized by molecular, genetic and biochemical approaches.

• The *ads1-D* mutant was compromised in both basal and nonhost resistance against microbial pathogens. Further, plant defence responses conferred by *RPS4* were also disabled in *ads1-D* plants. By contrast, depletion of *ADS1* transcripts by RNA-interference (RNAi) promoted basal disease resistance. Unexpectedly, *ads1-D* plants were found to constitutively accumulate reactive oxygen intermediates (ROIs). However, analysis of *ads1-D Arabidopsis thaliana respiratory burst oxidase* (*atrboh*) double and triple mutants indicated that an increase in ROIs did not impact *ads1-D*-mediated disease susceptibility.

• Our findings imply that *ADS1* negatively regulates the accumulation of the plant immune activator salicylic acid (SA) and cognate *Pathogenesis-Related 1* (*PR1*) gene expression. Collectively, these data highlight an important role for MATE proteins in the establishment of plant disease resistance.

## Introduction

Plants have evolved a complex repertoire of defence strategies that enable them to defend themselves against microbial infection. Nonhost disease resistance provides protection against the vast majority of potential pathogens (Nürnberger & Lipka, 2005). Typically, this is established by a series of preformed physical and chemical barriers (Haralampidis *et al.*, 2001) or by the induction of defence mechanisms in a nonspecific fashion by the recognition of microbial-associated molecular patterns (MAMPs) (Yun *et al.*, 2003; Nürnberger & Lipka, 2005; Zipfel & Robatzek, 2010). To overcome these bulwarks, potential pathogens have evolved strategies to tolerate, avoid or suppress these host defences. In response, plants have developed a gamut of resistance (*R*) gene products, which recognize, either directly or indirectly, pathogen-derived effector proteins (Dangl & Jones, 2001). These pathogen effectors, whose presence is detected by a given *R* gene product, have been termed avirulence (AVR) proteins, although their likely function is to aid pathogenesis on a susceptible host (Nomura *et al.*, 2006). The largest class of *R* genes

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encodes nucleotide-binding site (NBS) leucine-rich repeat (LRR) proteins, which can be divided into subgroups defined by the presence of either coiled-coil (CC) or Toll interleukin receptor (TIR) domains in their N-termini (Meyers *et al.*, 2003; Chini & Loake, 2005). Superimposed upon R-protein recognition is a further defence system that functions to restrain the growth of virulent pathogens. Designated basal resistance, this line of protection provides a supplementary barrier limiting the extent of infection (Glazebrook *et al.*, 1996). Together, these defence systems provide effective protection against attempted pathogen ingress, to an extent that disease is the exception amongst plant : pathogen interactions.

One of the most rapid defence mechanisms engaged following pathogen recognition is the oxidative burst, which constitutes the transient production of reactive oxygen intermediates (ROIs), primarily superoxide ($O_2^{\cdot-}$) and hydrogen peroxide ($H_2O_2$), at the site of attempted infection (Lamb & Dixon, 1997; Grant & Loake, 2000). This surge of ROIs is generated by the action of *Arabidopsis thaliana* respiratory burst oxidase (AtRBOH) proteins, and these NADPH oxidases are related to those that generate the pathogen-induced respiratory burst in mammalian phagocytes (Keller *et al.*, 1998; Torres *et al.*, 2002). While AtRBOH proteins comprise a relatively large gene family, AtRBOHD and AtRBOHF are thought to be responsible for ROI synthesis in leaves, following attempted pathogen ingress (Torres *et al.*, 2002).

The plant immune system activator, salicylic acid (SA), accumulates in plant tissue responding to attempted pathogen infection (Malamy *et al.*, 1990) and is essential for the establishment of some *R*-gene-mediated responses (Delaney *et al.*, 1994), systemic acquired resistance (SAR) (Gaffney *et al.*, 1993; Grant & Loake, 2007), basal defence (Delaney *et al.*, 1994) and also some cases of nonhost disease resistance (Feechan *et al.*, 2005). The accumulating evidence supports a role in which SA acts at multiple nodes in the defence signalling network, possibly by functioning as a signal amplifier (Fauth *et al.*, 1996; Mur *et al.*, 1996; Shirasu *et al.*, 1997). Nonexpresser of pathogenesis-related (PR) genes 1 (NPR1), is a key regulator of SA-based defence responses (Cao *et al.*, 1994). This ankyrin repeat protein is thought to be predominantly found in a cytoplasm-limited, homo-oligomeric complex through the formation of intermolecular disulphide bonds, as a default position in the absence of pathogen (Mou *et al.*, 2003). Following attempted microbial ingress, an SA-induced NPR1 oligomer-to-monomer conversion ensues, catalysed by thioredoxins (Tada *et al.*, 2008). NPR1 monomers are then free to move to the nucleus where they interact with transcriptional regulators such as TGA proteins to orchestrate expression of SA-dependent genes (Zhang *et al.*, 1999; Després *et al.*, 2000), which include those encoding PR proteins (Uknes *et al.*, 1992; Durrant & Dong, 2004).

T-DNA activation tagging is an efficient approach to generate dominant, gain-of-function mutations in plants (Grant *et al.*, 2003; Tani *et al.*, 2004) and we have employed this approach to identify novel regulators of plant disease resistance. Here, we describe the isolation of the *activated disease susceptibility1-Dominant* (*ads1-D*) mutant. This line exhibits enhanced disease susceptibility to a variety of pathogens. Further, an increase in SA and the subsequent accumulation of *PR1* transcripts is diminished in *ads1-D* plants. The *ADS1* gene is suppressed during the establishment of disease resistance and encodes a putative MATE transporter, a class of proteins whose number has undergone a major expansion in plants.

## Materials and Methods

### Activation tagging, plant material and pathogen infections

*Arabidopsis thaliana* (L.) Heynh. accession Col-0 and mutants derived from it were grown under 16 h of light at 22°C and 8 h of darkness at 18°C. *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 strains and *Pseudomonas syringae* pv. *phaseolicola* (*Psp*) NPS3121 were maintained and inoculated as described by Feechan *et al.* (2005). *Pst* strains were routinely inoculated at $10^5$ colony-forming units (cfu) $ml^{-1}$. *Botrytis cinerea* was grown and deployed as stated previously (Nurmberg *et al.*, 2007). *Blumeria graminis* f. sp. *tritici* (*Bgt*) WS14, obtained from Syngenta, was maintained on wheat cv Hereward.

### Determination of amounts of SA, ROIs and callose

Salicylic acid and SAG concentrations were determined as described (Aboul-Soud *et al.*, 2004). Briefly, six independent samples, each of 200 mg of leaf tissue from 5-wk-old plants, treated as described in the text, were ground in liquid nitrogen and subsequently extracted in 90–100% ethanol and vacuum-dried. The resulting pellets were resuspended in 5% trichloroacetic acid (TCA) and the mixture of ethylacetate : cyclopentane : isopropanol (50 : 50 : 1) solution. Fractionation of these resuspensions by simple centrifugation provided free SA (upper part) and SAG (lower part) fractions. The final filtered samples (50 µl injection) were subjected to high-performance liquid chromatography (HPLC, Dionex, Surrey, UK) with eluents of methanol and acetic acid.

To visualize ROIs *in situ*, 3,3-diaminobenzidine (DAB) staining was performed. Leaves were collected following indicated treatments and vacuum-infiltrated with the DAB solution. Leaves then were placed in a plastic box under high humidity until brown precipitate was observed (5–6 h) and then fixed with a solution of 3 : 1 : 1 ethanol : lactic acid : glycerol. Catalase effectively eliminated DAB

staining. Quantification of the staining was performed with Image J using 20 leaves similar to those presented (Yun *et al.*, 2003).

The extent of callose deposition was determined by aniline blue staining as described previously (Yun *et al.*, 2003). Briefly, following vacuum infiltration and boiling in lactophenol, leaves were incubated for 1 h, transferred to saturated chloral hydrate and agitated overnight. The following day, autofluorescence was quenched with toluidine blue. Samples were viewed by epifluorescence microscopy with excitation at 430 nm.

**Cell death measurements** The protocol for electrolyte leakage was adapted from Torres *et al.*, 2002). Briefly, 4-wk-old plants were injected with bacteria in 10 mM $MgCl_2$. Ten minutes after injection, 5-mm-diameter leaf discs were collected from the injected area and washed extensively with water for 10 min, and then 10 discs were placed in a Petri dish with 6 ml of water. Conductivity measurements (six replicates for each treatment) were taken over time by using a DiST WP (HANNA Instruments, Bedfordshire, UK) conductivity meter. The units of this measurement are $\mu S\ cm^{-1}$, where cm refers to the distance between electrodes.

**Phylogenetic tree construction** Sequence analyses and phylogenetic trees were carried out as previously described (Chini & Loake, 2005; Chini *et al.*, 2009). Briefly, alignments of protein sequences were generated using ClustalW (1.75) (http://sci.cnb.uam.es/Services/MolBio/clustalw) (Higgins *et al.*, 1996) and phenogram representation of the neighbour-joining tree of the MATE family was created by Phylodendron (http://iubio.bio.indiana.edu/treeapp/treeprint-form.html).

### Molecular biology procedures

DNA sequences flanking the site of T-DNA insertion in *ads1-D* plants were obtained with GenomeWalker (GenomeWalker™ Kits, Clontech-Takara Bio Europe, Saint-Germain-En-Laye, France) and subsequently sequenced. RNA blot hybridization was carried out using probes of defence-related genes generated as described by Murray *et al.*, 2002. For semiquantitative reverse transcription polymerase chain reaction (RT-PCR), DNA in RNA samples was removed with DNA-free kit (Ambion) or acidic solution of 5 : 1 phenol : chloroform and the resulting RNA reverse-transcribed into cDNA with Moloney Murine Virus (MLV) reverse transcriptase (Promega).

For transgenic reconstitution, the genomic sequence of *ADS1* was PCR-amplified using the following primers: forward primer, 5′-GGAATTCGAGAAGAGAGAAGCAGCACCA-3′; reverse primer, 5′-CGGGATCCTCATAAGGGCATCGGAAAAA-3′. The PCR fragment was digested with *Eco*RI and *Bam*HI and cloned into the *Eco*RI- and *Bam*HI-digested pART7 downstream of the *35S* promoter. A DNA fragment containing *35S*::*ADS1* was prepared by *Not*I digestion of recombinant pART7 and ligated into *Not*I-digested pGreen0229. The resulting construct was transformed into wild-type Col-0 plants as described previously (Clough & Bent, 1998). Transgenic lines overexpressing *At4g2915*0 were generated in a similar fashion following amplification of the cDNA sequence using the following primers: forward primer, 5′-ATCGAATTCATGAGAAAGAATCTCA-3′; reverse primer, 5′-ATCGGATCCTCACCAACGCATCCTA-3′. In each case, three independent transgenic plants were examined and results presented for a representative line.

To generate a genetic construct for RNAi of *ADS1*, the third intron of the *Arabidopsis* actin 11 gene (GenBank *ATU27981*; TAIR At3g12110) was selected for the intron containing intermediate construct. The intron was amplified by PCR and the resulting product was digested with *Sna*BI and *Eco*RI and subsequently cloned into *Eco*RV- and *Eco*RI-digested pBluescript II SK+ to yield the intermediate construct pSK-int. To clone the sequence encoding the inverted-repeat RNA into the pSK-int intermediate vector, a 345 bp fragment of *ADS1* coding sequence was cloned into the 5′ and 3′ arms. The fragment with inverted-repeated *ADS1* sequences was subsequently obtained and inserted into pART7 downstream of the *35S* promoter, and subsequently this construct was transferred into pGreen0229 by *Not*I digestion. Three transgenic lines were chosen for further analysis and each of these lines generated similar results. For semiquantitative RT-PCR, DNA in RNA samples was removed with DNA-free kit (Ambion) or an acidic solution of 5 : 1 phenol : chloroform and the resulting RNA was reverse-transcribed into cDNA with MLV reverse transcriptase (Promega). RT-PCR was undertaken using 21 cycles.

## Results

### Identification of *ads1-D* by activation tagging

A large activation T-DNA tagged population of Col-0 *Arabidopsis* plants (Grant *et al.*, 2003; Chini *et al.*, 2004) was screened for perturbed defence responses. Approximately 5000 T1 lines from this population were challenged with virulent *Pst*DC3000 by pressure infiltration and scored for increased susceptibility towards this bacterial pathogen. The first mutant identified from this screen was designated *ads1-D*. Further infection assays in T2 plants confirmed that *Pst*DC3000 reached a higher titre in this line relative to wild-type plants (Fig. 1a). Disease susceptibility towards *Pst*DC3000 cosegregated with ammonium glufosinate herbicide resistance encoded within the T-DNA insert. Approximately 75% (226/297) of these $T_2$ progeny retained the mutant phenotype and were herbicide-

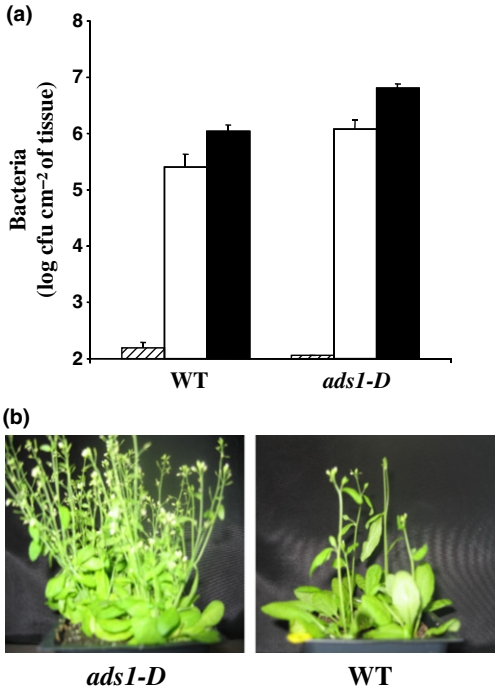


**Fig. 1** Identification of *ADS1* by activation tagging. (a) Basal resistance against *Pst*DC3000 is compromised in *ads1-D* plants. Leaves of 4-wk-old *Arabidopsis* plants were infiltrated with *Pst*DC3000 and bacterial titres determined in triplicate at 0 (hatched bar), 3 (white bar) and 5 (black bar) d postinoculation (dpi). Data points are the mean of three technical replicates (± SD). Student's *t*-test confirmed significant differences at $P = 0.05$ between the growth of *Pst*DC3000 at 3 and 5 d (dpi) in *ads1-D* relative to wild-type (WT) plants. This experiment was repeated with similar results. (b) The morphological phenotype of *ads1-D* plants relative to wild-type Col-0. The *ads1-D* line is reduced in stature and exhibits a loss of apical dominance.

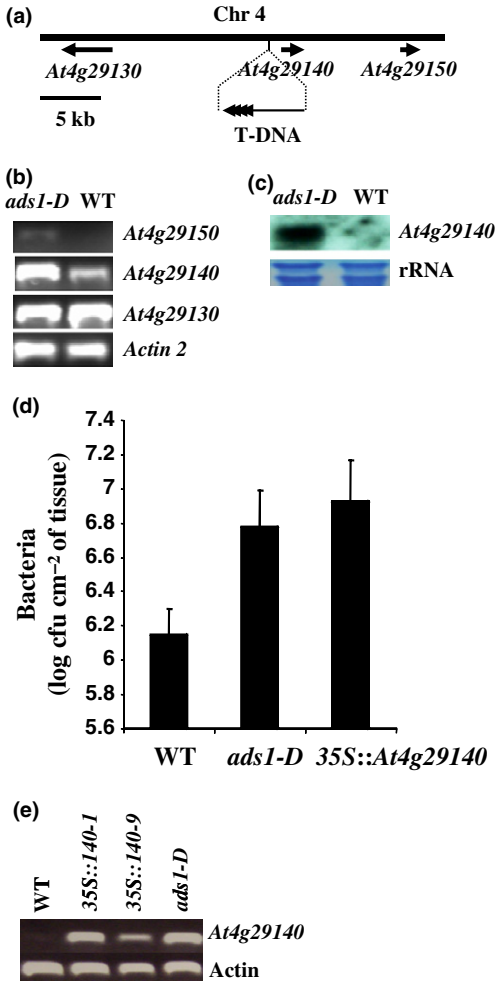


**Fig. 2** *ADS1* encodes a multidrug and toxic compound extrusion (MATE) transport protein. (a) Schematic showing the position of the T-DNA insert within the *ads1-D* mutant relative to flanking genes. The thick black line denotes the DNA sequence on chromosome 4. Arrows indicate the position and transcriptional direction of the stated genes. Four repeated arrows in tandem within the T-DNA show the position and direction of the *35S* enhancer sequences. (b) Expression of genes adjacent to the inserted T-DNA in the *ads1-D* line relative to that in wild-type (WT) plants, determined by 30 cycles of reverse transcription polymerase chain reaction (RT-PCR). (c) Expression of *At4g29140* in *ads1-D* plants relative to WT measured by northern blot analysis. (d) Titre of *Pst*DC3000 in a representative *Arabidopsis* line containing a *35S::At4g29140* transgene, *ads1-D* and WT plants. Data points are the means of three replicate samples (± SD). To better present the differences in bacterial growth among *ads1-D*, *35S::At4g29140* and WT plants, the *y*-axis scale does not start from zero. (e) Northern blot analysis of *At4g29140* in two representative *Arabidopsis* lines containing a *35S::At4g29140* transgene relative to WT and *ads1-D* plants.

resistant, whereas all plants exhibiting a wild-type phenotype, *c.* 25% (71/297), were herbicide-susceptible. The chi-squared value of $T_2$ plants ($\chi^2 = 0.194$; $P = 0.01$ with one degree of freedom) showed a herbicide resistance : susceptibility ratio that did not deviate significantly from the expected 3 : 1 ratio with a confidence of 99%. Collectively, these results suggested that a single dominant effect was responsible for this phenotype. *ads1-D* plants were also reduced in stature, displayed a conspicuous loss-of-apical dominance (Fig. 1b) and flowering was earlier in long-day conditions relative to Col-0. In addition, the fertility of this mutant was also reduced.

To identify *ADS1*, the adjacent genome DNA fragments of the T-DNA were obtained by genome walking and subsequently sequenced. The results showed that the single T-DNA insert was located on chromosome 4 between genes *At4g29130* and *At4g29140* (Fig. 2a). These genes encoded hexokinase 1 and a MATE-transporter, respectively. To help identify which gene corresponds to *ADS1*, we determined the expression of genes around the T-DNA insert by RT-PCR. The presence of CaMV*35S* enhancers within the T-DNA did not up-regulate the expression of the hexokinase 1 gene (Fig. 2b). By contrast, transcripts of *At4g2914*0 and *At4g2915*0, which encoded a calmodulin-binding protein, were strongly and weakly up-regulated, respectively, in *ads1-D* plants relative to wild-type. Further, the enhanced

expression of *At4g2914*0 in the *ads1-D* line was confirmed by northern blot analysis (Fig. 2c). Collectively, these data suggest that overexpression of either *At4g2914*0 or *At4g2915*0, or both, may contribute to the *ads1-D* phenotype. To discriminate between these possibilities, full-length cDNA clones were generated for these genes and subsequently cloned downstream of *35S* enhancer sequences. The resulting constructs were then transformed individually into wild-type Col-0 plants. Analysis of the resulting transgenic lines revealed that *35S::4g29140* reconstituted the development phenotype to *ads1-D* plants and significantly only this line showed increased susceptibility to *Pst*DC3000 (Fig. 2d; Supporting Information, Fig. S1). Analysis of *At4g29140* gene expression in these plants confirmed this candidate gene was overexpressed in each of these *Arabidopsis* lines, as exemplified in Fig. 2(e). Taken together, these data imply that ADS1 is a putative MATE transporter encoded by *At4g29140*.

The *ADS1* MATE gene is 1599 bp in length, possesses one exon and encodes a predicted protein of 532 amino acids. *Arabidopsis* possesses 55 full-length MATE sequences. However, no apparent consensus sequence is conserved within these proteins; rather, MATE transporters share a relatively low overall sequence similarity. Following alignment of these sequences with ClustalW, we generated a phylogenetic tree using Phylodendron. This analysis placed ADS1 within a clade containing only one other protein, encoded by At5G19700 (Fig. 3a). ADS1 and At5G19700 share only 63% identity and 76% similarity. Further, *in silico* analysis using HMMTOP transmembrane topology prediction (Tusnady & Simon, 2001) suggested that ADS1 possesses 12 transmembrane regions (Fig. 3b,c).

## Response of *ads1-D* plants to other pathogens

*ads1-D* plants were challenged with *Pst*DC3000 expressing *avrRPS4* which is recognized by the TIR-NBS-LRR gene product RPS4 (Gassmann *et al.*, 1999). The difference between the growth of this avirulent strain and *Pst*DC3000 is less in the *ads1-D* mutant than observed for wild-type (Fig. 4a). Thus, RPS4-mediated disease resistance is compromised in the *ads1-D* line.

To explore if *ads1-D* plants are susceptible to other pathogens in addition to virulent and avirulent strains of *Pst*DC3000, we challenged this line with *P. syringae* pv. *phaseolicola* (*Psp*) NPS3121. *Arabiodopsis* is ordinarily a nonhost for this bacterial pathogen (Feechan *et al.*, 2005). The titre of *Psp*NPS3121 in *ads1-D* plants was markedly greater than that in the wild-type Col-0 line (Fig. 4b). Therefore, *ads1-D* also compromised nonhost resistance against this pathogen. To investigate this observation further, we monitored the profile of callose deposition, a marker for the expression of resistance at the cell wall, in *ads1-D*, *35S::At4g29140* and wild-type plants. The extent of callose deposition was both delayed and reduced in the *ads1-D* line relative to wild-type plants following attempted *Psp*NPS3121 ingress (Figs 4c, S2). We also determined the response of these lines to *Bgt*, a nonadapted fungal pathogen for *Arabidopsis*. Both the *ads1-D* and *35S::At4g29140* lines supported a greater extent of *Bgt* haustorial formation relative to that observed on wild-type plants (Fig. 4d). Further, attempted *Bgt* infection triggered less callose deposition on *ads1-D* plants in comparison to the wild-type line (Figs 4e, S3).

To investigate the response of the *ads1-D* line to a necrotrophic pathogen, leaves of *ads1-D* and control plants were challenged with *Botrytis cinerea* PJH2 (Nurmberg *et al.*, 2007). The *ads1-D* line did not exhibit enhanced disease susceptibility towards this pathogen (Fig. S4).

## Depletion of *ADS1* gene expression conveys disease resistance

As overexpression of *ADS1* leads to enhanced disease susceptibility we investigated if reduced *ADS1* transcript accumulation could convey increased disease resistance. Unfortunately, there was no available T-DNA insertion mutant for *ADS1* from any of the *Arabidopsis* mutant stock centres. Therefore, we employed a transgenic approach using RNAi to deplete *ADS1* transcript abundance. Transgenic lines in which *ADS1* gene expression was robustly reduced were selected for further experiments (Fig. 5a). These lines resembled wild-type Col-0 plants. Plants with reduced abundances of *ADS1* transcripts were challenged with virulent *Pst*DC3000 and scored for the expression of basal disease resistance. Depletion of *ADS1* transcripts by RNAi supported a decreased titre of this bacterial pathogen relative to wild-type plants, shown for a representative line designated *rADS1* (Figs 5b, S5).

## *ads1-D* plants exhibit AtRBOH-dependent $H_2O_2$ accumulation

A key feature of the plant defence response is the rapid synthesis of ROIs such as $H_2O_2$ (Lamb & Dixon, 1997; Grant & Loake, 2000). We therefore determined the extent of ROI accumulation in the *ads1-D* line. *Pst*DC3000(*avrB*) challenged and unchallenged plants were stained with DAB, which marks $H_2O_2$ with a brown precipitate (Yun *et al.*, 2003). Unexpectedly, *ads1-D* plants showed a significant increase in ROIs relative to wild-type unchallenged plants (Figs 6a, S6). Thus, the *ads1-D* line accumulates ROIs even in the absence of pathogen challenge. The oxidative burst is thought to be driven predominantly through the activity of AtRBOHD and, to a lesser extent, AtRBOHF in *Arabidopsis* (Torres *et al.*, 2002). To investigate if these enzymes are the source of ROI production in *ads1-D* plants, we generated *atrboh* double and triple mutants. In the

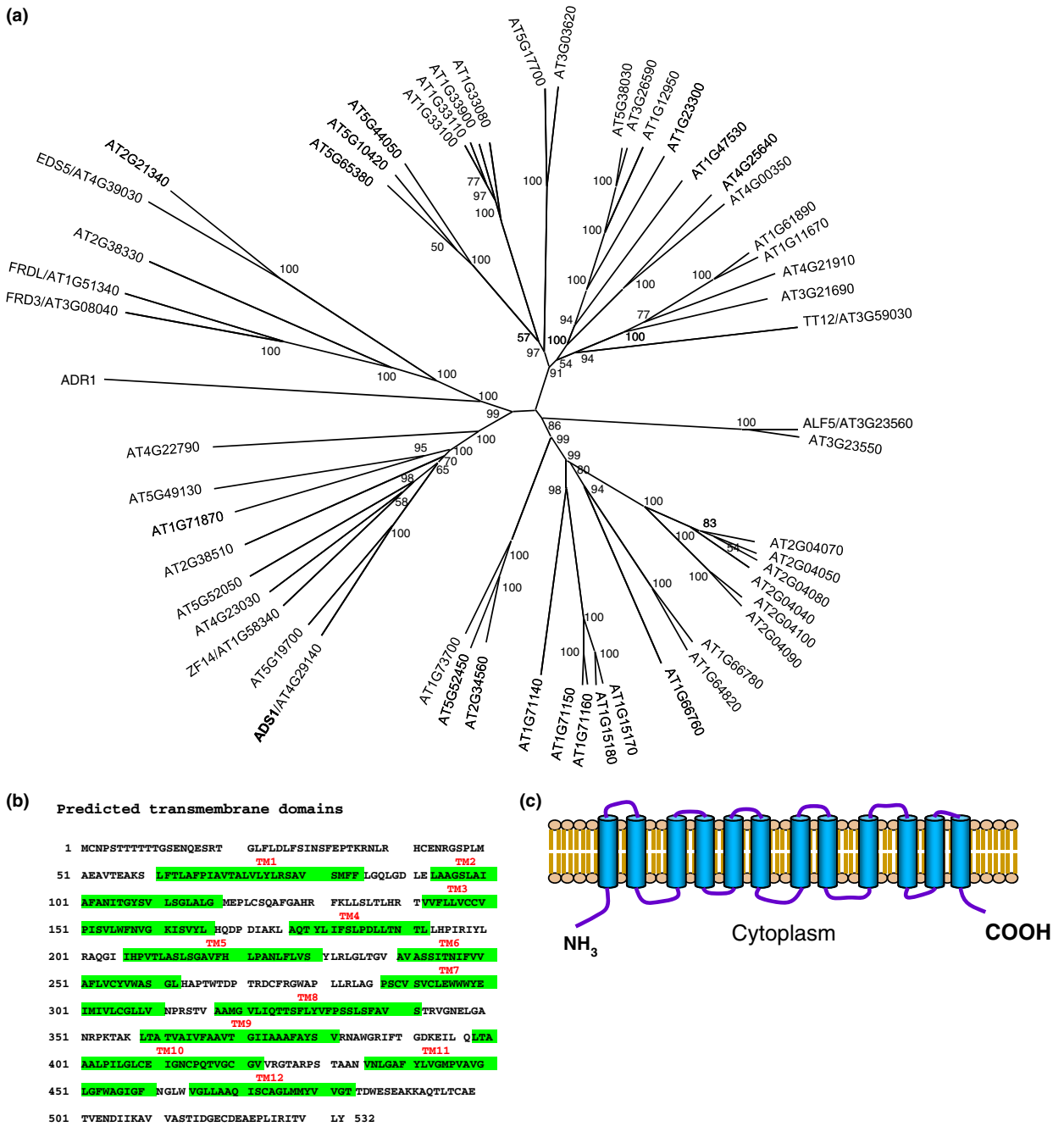


**Fig. 3** *ADS1* is a member of the multidrug and toxic compound extrusion (MATE) transporter protein family. (a) Phylogenetic tree of *Arabidopsis* MATE proteins. Phenogram representation of the neighbour-joining tree for the 55 full-length MATE sequences and the ADR1 protein (Chini *et al.*, 2004) as outgroup. Sequence alignment was generated using ClustalW and the tree was created by Phylodendron. Branch lengths are proportional to the estimated evolutionary distance. Bootstrap values are included. (b) *In silico* analysis using HMMTOP (Tusnady & Simon, 2001) indicates that ADS1 is an integral membrane protein which is predicted to possess 12 transmembrane spanning domains. (c) Topology of the MATE transporter ADS1 predicted by HMMTOP.

absence of pathogen challenge, the amounts of ROIs in the *ads1-D atrbohD* line were largely indistinguishable from those in *ads1-D* plants (Figs 6b, S7). By contrast, *ads1-D atrbohF* plants, which resembled *ads1-D* plants, exhibited a striking decrease in ROIs. Furthermore, a similar result was obtained with the *ads1-D atrbohF atrbohD* triple mutant line (Figs 6b, S7). Collectively, these results imply that AtRBOHF, rather than AtRBOHD, is the major source of ROI generation in unchallenged *ads1-D* plants.

To determine if this prior ROI accumulation had an impact on the defence response, we challenged the *ads1-D atrbohF* double mutant with *Pst*DC3000. The titre of

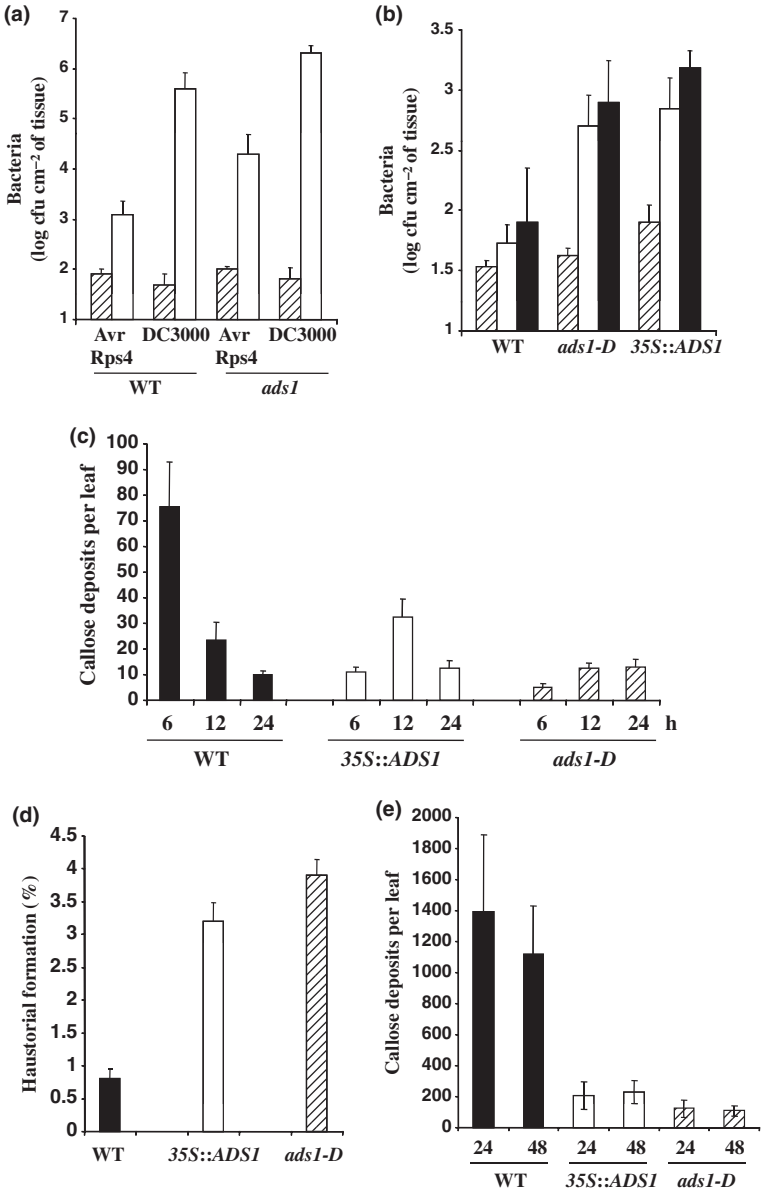


**Fig. 4** *R*-gene-mediated protection and nonhost resistance in *ads1-D* plants. (a) Leaves of 4-wk-old Arabidopsis wild-type (WT) or *ads1-D* plants were infiltrated with either *Pst*DC3000(*avrRps4*) or *Pst*DC3000 and bacterial titres determined at 0 (hatched bar) and 3 (white bar) d postinoculation (dpi). (b) Leaves of 4-wk-old *ads1-D*, *35S::ADS1* and WT plants were infiltrated with *Psp* NPS3121 at $1.5 \times 10^5$ cfu $ml^{-1}$ and bacterial titres determined at 0 (hatched bars), 3 (white bars) and 5 (black bars) dpi. To better present the differences in bacterial growth between *ads1-D*, *35S::ADS1* and WT plants, the *y*-axis scale does not start from zero. Student's *t*-test confirmed significant differences at $P = 0.05$ between bacterial growth in *ads1* lines relative to WT plants in (a) and (c) and also between the extent of discrepancies in bacterial titre among *Pst*DC3000(*avrRps4*)- or *Pst*DC3000-infiltrated WT and *ads1* plants. (c) Extent of callose deposition in *ads1-D*, *35S::ADS1* and WT plants following attempted *Psp* NPS3121 ingress at the times indicated. (d) Determination of the magnitude of haustorial formation in *ads1-D*, *35S::ADS1* and WT plants at 48 h postinoculation with *Blumeria graminis* f. sp. *tritici*. (e) Extent of callose deposition in *ads1-D*, *35S::ADS1* and WT plants following attempted *Bgt* ingress at the times indicated. All data points are the means of three technical replicates (± SD). All experiments were repeated at least once with similar results.

*Pst*DC3000 growth in this line was similar to that of *ads1-D* plants (Fig. 6c). Thus, ROI accumulation before pathogen challenge does not modulate the expression of disease resistance in the *ads1-D* line.

## *ads1-D* plants are compromised in SA synthesis and signalling

To identify the molecular mechanism underpinning the action of *ADS1* in the plant defence response, we investigated the expression of key defence marker genes. The accumulation of transcripts for *PR1*, a marker for SA-based responses (Uknes *et al.*, 1992), was significantly delayed in *ads1-D* plants challenged with *Pst*DC3000 relative to wild-type (Fig. 7a). *PR1* expression was first detected in wild-type plants at 12 h postinoculation (hpi). However, transcripts corresponding to this gene were not detected in *ads1-D* plants until 48 hpi. These results prompted us to determine the concentrations of SA in unchallenged plants and the amount of this immune activator in the *ads1-D* line and *35S::ADS1* plants over time following inoculation with *Pst*DC3000. There was a marked decrease in the concentrations of SA in mutant and transgenic *ADS1* lines relative to unchallenged wild-type. Further, following *Pst*DC3000 infection, the SA concentration in these lines was conspicuously less than that which accumulated in wild-type plants inoculated with this pathogen at 24 and 48 hpi (Fig. 7b). A similar trend was observed for the β-glucoside of SA (SAG) (Fig. 7c). Collectively, these data imply that both pathogen-challenged and untreated *ads1-D* plants accumu-

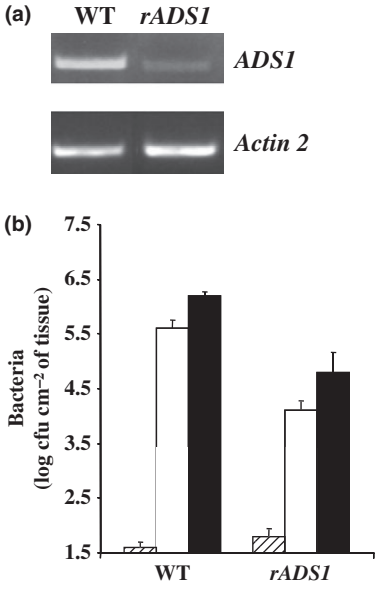


**Fig. 5** Depletion of *ADS1* transcript accumulation promotes basal resistance. (a) Reverse transcription polymerase chain reaction (RT-PCR) analysis of the *rADS1* line showing RNAi of *ADS1* transcript accumulation. (b) Growth of *Pst*DC3000 in wild-type (WT) and *rADS1* at 0 (hatched bar), 3 (white bar) and 5 (black bar) d postinoculation (dpi). To better present the differences in bacterial growth between the *rADS1* line and wild-type plants, the *y*-axis scale does not start from zero. Data points are the averages of three technical replicates (± SD). Student's *t*-test confirmed significant differences at $P = 0.05$ between bacterial growth in the *rADS1* line relative to WT plants. These experiments were repeated with similar results. DAB, 3,3-diaminobenzidine.

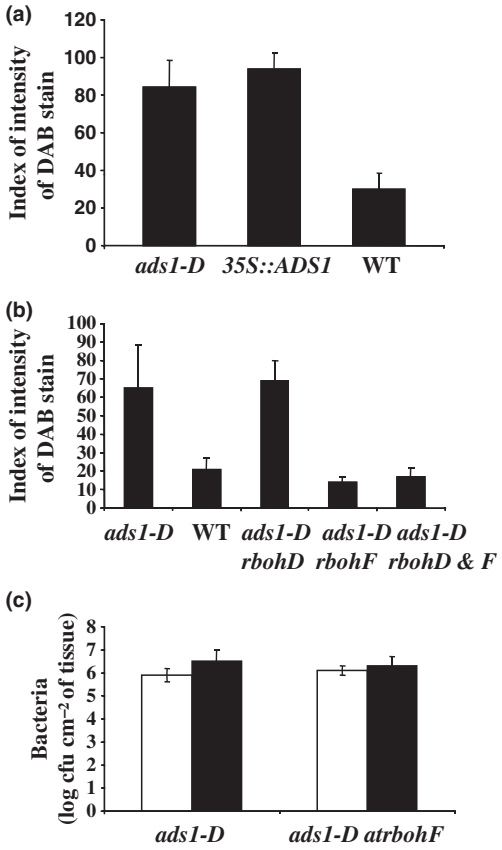


**Fig. 6** AtRBOH-dependent reactive oxygen intermediate (ROI) accumulation in *ads1-D* plants. (a) Determination of basal $H_2O_2$ concentrations in the indicated plant lines by DAB staining. (b) Quantification of base line ROI accumulation in the stated plant genotypes determined by DAB staining. (c) Titre of *Pst*DC3000 in *ads1-D* and *ads1-D atrbohF* plants at 3 (white bar) and 5 (black bar) d post inoculation (dpi). These experiments were repeated three times with similar results. Data points are the average of three technical replicates (± SD).

late lower concentrations of both SA and SAG relative to wild-type. To investigate if the reduction of SA accumulation in *ads1-D* plants was responsible for their increased susceptibility towards *Pst*DC3000, we sprayed mutant and transgenic *ADS1* lines with SA and subsequently scored the growth of this pathogen within these plants. Comparison of the relative difference between mock and SA treatment for each plant genotype implied that previous treatment with this immune activator reduced the titre of *Pst*DC3000 in *ads1-D* and *35S::ADS1* plants in a similar fashion to that of wild-type (Fig. 7d–f).

## Discussion

Our data identify ADS1 as a putative member of the MATE protein family. Multidrug transporters function in the transport of toxic metabolites or xenobiotics across membranes in both prokaryotic and eukaryotic cells. These proteins have been classified into six families (Omote *et al.*, 2006), including the MATE (Putman *et al.*, 2000) and ATP-binding cassette (ABC) (Brown *et al.*, 1999) transport families. No apparent consensus sequence is conserved in all MATE proteins; however, all these transporters share *c.* 40% sequence similarity. These proteins may couple the transport of their target molecule with an electrochemical gradient of $H^+$ or $Na^+$ ions across the membrane (Omote *et al.*, 2006). In contrast to the relatively small number of MATE genes found in bacterial and animal species, this gene family has undergone a remarkable expansion in plants, thus highlighting the importance of MATE proteins in this kingdom. To date, MATE transporters in plant species appear to be largely involved in the detoxification of endogenous secondary metabolites (Gomez *et al.*, 2009), xenobiotics (Diener *et al.*, 2001) and by extension from animal studies (Otsuka *et al.*, 2005), possibly steroids, likely utilizing an $H^+$ exchange mechanism. In addition, plant MATE transporters have been shown to function in the synthesis of natural products such as proanthocyanidins (condensed tannins) (Zho & Dixon, 2009).

The *ads1-D* line does not appear to exhibit increased susceptibility towards *B. cinerea* PJH2, suggesting *ADS1* function does not have an impact on the defence response

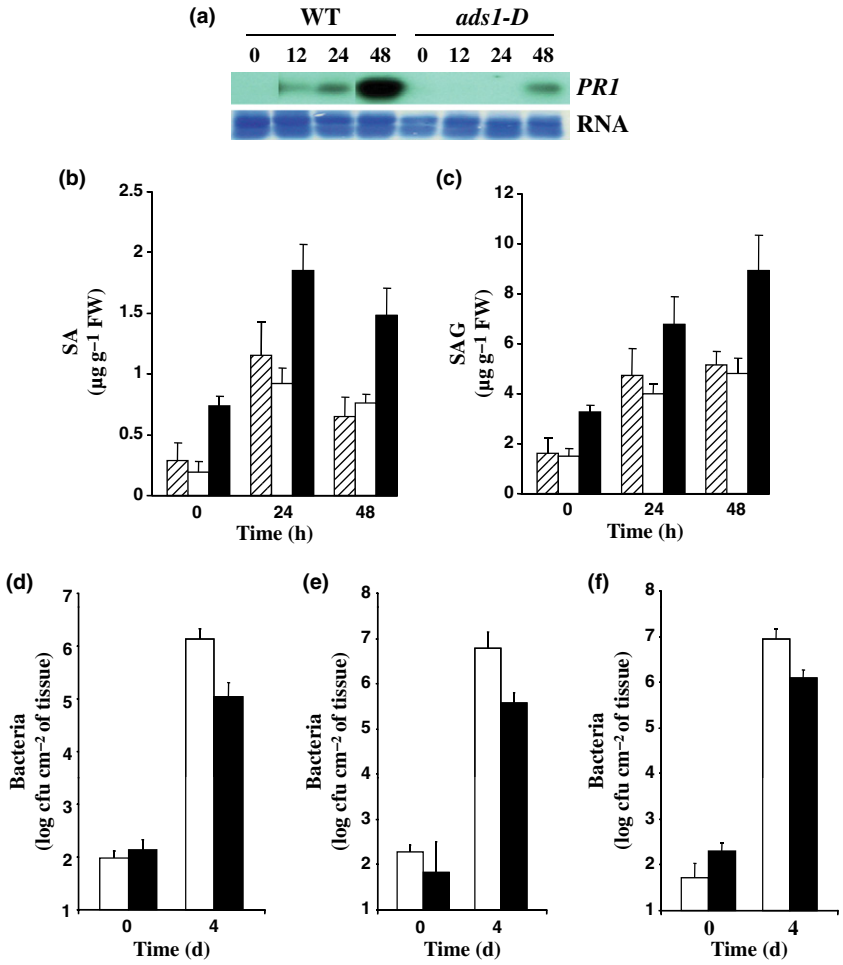


**Fig. 7** Salicylic acid (SA) synthesis and signalling are modulated in *ads1-D* plants. (a) Northern blot analysis of *PR1* gene expression over time following challenge with *Pst*DC3000. The bands corresponding to wild-type (WT) 12 and 24 h time points are derived from an independent blot. (b) SA concentrations at 24 and 48 h postinfection (hpi) with *Pst*DC3000; (c) increase of β-glucoside of SA (SAG) at 24 and 48 hpi with *Pst*DC3000 (*ads1-D*, hatched bars; *35S::ADS1*, white bars; WT, black bars). (d–f) Titre of *Pst*DC3000 in mock (open bars) and SA-treated (closed bars) plants of the given genotypes at 0 and 4 d postinoculation (dpi). Plants were treated with 0.1 mM SA 3 d before inoculation. To better present the differences in bacterial growth between *ads1-D*, *35S::ADS1* and wild-type plants, the *y*-axis scale does not start from zero. Data points are the averages of three technical replicates (± SD). These experiments were repeated with similar results. Student's *t*-test confirmed significant differences at $P = 0.05$ between SA accumulation in *ads1* lines relative to WT plants (b, c) and bacterial growth between mock and SA-treated plants (d–f).

against necrotrophic pathogens. However, activation tagging of *ADS1* conveyed increased disease susceptibility towards *Pst*DC3000. Thus, *ADS1* overexpression compromises basal disease resistance, at least against this bacterial pathogen. By contrast, depletion of *ADS1* transcripts established increased protection against *Pst*DC3000. Furthermore, *ads1-D* plants also exhibited increased susceptibility towards *Pst*DC3000 expressing *avrRps4*. The expression of *R*-gene-mediated resistance conveyed by at least one TIR subclass NBS-LRR protein is therefore diminished in *ads1-D* plants. Also, overexpression of *ADS1* supported increased growth of *Psp*NPS3121. Hence, the *ads1-D* line is also disabled in nonhost resistance. Conversely, ADS1 function did not have an impact on the defence response against the necrotrophic pathogen *B. cinerea*. Therefore, ADS1 activity may control processes integral to the interaction of *Arabidopsis* with *P. syringae* species.

In addition to the defence response, overexpression of *ADS1* also had an impact on growth and development. Thus, disabled disease resistance in *ads1-D* plants might be an indirect consequence resulting from these morphological changes. However, depletion of *ADS1* transcripts enhanced basal resistance in the absence of growth defects.

Further, addition of SA to *ads1-D* plants promoted resistance in a similar fashion to SA-treated wild-type plants, despite the morphological changes present in the *ads1-D* line. Therefore, depleted SA concentrations might largely account for this defence-related phenotype. Collectively, these findings imply that *ADS1* is a negative regulator of basal, *R*-gene-mediated and nonhost resistance.

Our pathology data relating to *ads1-D* are consistent with the reported profile of *ADS1* transcript accumulation. Mining of information within publicly available databases suggested that *ADS1* expression is repressed during the establishment of disease resistance against a diverse series of biotrophic and hemibiotrophic pathogens, including some for which *Arabidopsis* is a nonhost (Tao *et al.*, 2003; Stein *et al.*, 2006; Table S1). Further, *ADS1* transcripts are also diminished following treatment with the plant immune system activator, SA (Wang *et al.*, 2005). This is consistent with the notion that overexpression of *ADS1* antagonizes SA signalling and cognate SA-dependent gene expression.

Methyl-jasmonate (Me-JA), in the presence of ethylene (ET), is a key positive regulator of defence responses against necrotrophic pathogens (Penninckx *et al.*, 1996; Grant &

Jones, 2009). Me-JA is known to blunt SA-dependent defence signalling and some bacterial species exploit this fact by synthesizing coronatine, a Me-JA mimic, to aid pathogenesis (Brooks *et al.*, 2005). Consistent with these observations, the expression of *ADS1*, a negative regulator of SA responses, is induced by both Me-JA and ET (Table S1; Genevestigator at http://www.arabidopsis.org/).

Another MATE transporter, enhanced disease susceptibility 5 (EDS5) (Nawrath *et al.*, 2002), has previously been shown to function in plant–pathogen interactions. However, ADS1 and EDS5 share relatively limited sequence homology, having between 12% identical amino acids and 27% homologous amino acids and also locate to distinct groups within the phylogenetic tree for this gene family. Loss-of-function mutations in *EDS5* compromised SA accumulation and an increase in *PR1* transcripts. Consequently, this *Arabidopsis* mutant was found to be compromised in basal disease resistance and also in protection mediated by some *R* genes. Further, *EDS5* was found to be pathogen- and SA-inducible (Nawrath & Metraux, 1999). More recently, this MATE protein has also been implicated in viral resistance, as overexpression of *EDS5* resulted in increased SA accumulation and promoted basal protection against Cucumber mosaic and Turnip crinkle viruses (Ishihara *et al.*, 2008). Therefore, *Arabidopsis* appears to possess MATE proteins that can function as either positive or negative regulators of disease resistance.

ATP-binding cassette proteins, another class of the multidrug transporter super family, have also been implicated in the plant defence response. The absence of *PENETRATION3* (*PEN3*) function, which encodes an ABC transporter that localizes to infection sites, reduced penetration resistance against *Bgh* (Stein *et al.*, 2006), suggesting this protein contributes to defences at the cell wall and intracellularly. Recently, *LEAF RUST 34* (*Lr34*), which has conveyed resistance to wheat leaf rust, stripe rust and powdery mildew for over 50 yr, was identified and also found to encode an ABC transport protein (Krattinger *et al.*, 2009).

A feature of the *ads1-D* line was its accumulation of ROIs relative to wild-type plants in the absence of pathogen challenge. The engagement of the oxidative burst is thought to be a key component of the defence response, with ROIs functioning as both antimicrobial effectors and signalling molecules (Lamb & Dixon, 1997; Grant *et al.*, 2000). However, somewhat counterintuitively, *ads1* plants, which accumulated increased amounts of ROIs in the absence of pathogen challenge, exhibited increased disease susceptibility. This accumulation of ROIs might reflect inappropriate activation of AtRBOHF, as a loss-of-function mutation in the corresponding gene blunted this phenotype. With regard to its enhanced disease susceptibility, the *ads1-D* line resembles *A. thaliana S-nitrosogluthathione reductase 1-3* (*atgsnor1-3*), which leads to increased nitric oxide (NO) and S-nitrosothiols (SNOs), resulting in nitrosative stress, but nevertheless this mutation conveys broad-spectrum susceptibility (Feechan *et al.*, 2005; Wang *et al.*, 2009). This may reflect a requirement for the parallel engagement of the oxidative and nitrosative burst in these lines in order to orchestrate an effective defence response. In this context, NO and ROIs are thought to act cooperatively to drive the development of cell death associated with the hypersensitive response (Delledonne *et al.*, 2001).

Reactive oxygen intermediates are thought to promote the oxidative cross-linking of NPR1 monomers blocking their translocation to the nucleus and the subsequent NPR1-dependent activation of *PR* gene expression (Mou *et al.*, 2003). Thus, increased ROIs in *ads1-D* plants might blunt defence signalling through NPR1. However, this is unlikely, because *ads1-D atrbohF* plants, which do not accrue ROIs, exhibit similar amounts of pathogen susceptibility to the *ads1-D* line. Hence, the defence-related phenotype of *ads1-D* plants may not be connected to spurious ROI synthesis.

Multidrug and toxic compound extrusion proteins are thought to couple transport of their target molecules across a membrane with an electrochemical gradient of $H^+$ or $Na^+$ ions, requiring the action of a plasma membrane $H^+$-ATPase, a vacuolar $H^+$-ATPase or a vacuolar $H^+$-pyrophosphatase. Hence, overexpression of ADS1 might deplete the activity of a given member of one of these classes of protein that is also necessary for the transport of one or more key defence metabolites. Subsequently, the diminished amounts of this molecule may become limiting for SA synthesis and the timely expression of *PR* genes.

Our findings imply that enhanced expression of *ADS1* antagonizes the defence response. Thus, distinct members of the MATE transporter protein family can function as negative in addition to positive regulators of disease resistance. Recent data have also highlighted a potential role for ABC transporters in plant–pathogen interactions. Screening for *Arabidopsis* mutants compromised in resistance to *B. graminis* f. sp. *hordei*, identified PENETRATION3 (PEN3), a highly expressed putative ABC transporter. Analysis of *pen3* mutants suggested that PEN3 contributed to defences at the cell wall (Collins *et al.*, 2003; Stein *et al.*, 2006). Further, *Lr34*, which has been utilized to provide resistance against leaf rust, stripe rust, and powdery mildew, has been shown to encode a putative ABC transport protein. *Lr34* is thought to mediate a senescence-like process at the edges and tips of flag leaves (Krattinger *et al.*, 2009). Collectively, members of the multidrug transporter super family are therefore emerging as important players in the establishment of plant disease resistance.

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## Supporting Information

Additional supporting information may be found in the online version of this article.

**Fig. S1** Overexpression of *At4g29150* does not enhance resistance against *Pst*DC3000.

**Fig. S2** Callose deposition in the *ads1-D* line relative to wild-type plants at 6 h postinoculation of *Psp*NPS3121.

**Fig. S3** Callose deposition in the *ads1-D* line relative to wild-type plants at 48 h postinoculation of *Bgt*.

**Fig. S4** Challenge of the given *Arabidopsis* genotypes with *Botrytis cinerea*.

**Fig. S5** Depletion of *ADS1* transcript accumulation promotes basal resistance.

**Fig. S6** Reactive oxygen intermediate (ROI) accumulation in *ads1-D*, *35S::ADS1* and wild-type plants in the absence of pathogen challenge.

**Fig. S7** Reactive oxygen intermediate (ROI) accumulation in *ads1-D* double and triple mutants.

**Table S1** Change in *ADS1* transcript abundance during attempted pathogen infection or in response to defence-related cues or abiotic stresses

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